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Naturally acquired immunity against immature *Plasmodium falciparum* gametocytes

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31 **One sentence Summary:** Naturally acquired immune responses target infected red blood cell surface
32 antigens of immature malaria transmission stages.

33

Abstract

The recent decline in global malaria burden has stimulated efforts towards *Plasmodium falciparum* elimination. Understanding the biology of malaria transmission stages may provide opportunities to reduce or prevent onward transmission to mosquitoes. Immature *P. falciparum* transmission stages, termed stage I-IV gametocytes, sequester in human bone marrow before release into the circulation as mature stage V gametocytes. This process likely involves interactions between host receptors and potentially immunogenic adhesins on the infected red blood cell (iRBC) surface. Here we developed a flow cytometry assay to examine immune recognition of live gametocytes of different developmental stages by naturally exposed Malawians. We identified strong antibody recognition of the earliest immature gametocyte-iRBCs (giRBCs) but not mature, stage V giRBCs. Candidate surface antigens (n=30), most of them shared between asexual- and gametocyte-iRBCs and others enriched in giRBCs, were identified by mass spectrometry and mouse immunizations, as well as correlations between responses by proteome microarray and flow cytometry. Naturally acquired responses to a subset of candidate antigens were associated with reduced asexual and gametocyte density, and plasma samples from malaria-infected individuals were able to induce immune clearance of giRBC *in vitro*. Infected RBC surface expression of 6 select candidate antigens was validated using specific antibodies in fluorescent microscopy and flow cytometry experiments, and genetic analysis revealed a subset with minimal variation across strains. Our data demonstrate that humoral immune responses to immature giRBCs and shared iRBC antigens are naturally acquired following malaria exposure. These humoral immune responses may have consequences for malaria transmission potential by clearing developing gametocytes, which could be leveraged for malaria intervention.

Introduction

Plasmodium falciparum malaria morbidity and mortality has decreased substantially in the last decade (1). These recent gains are threatened by the spread of artemisinin-resistant parasites (2) and insecticide-resistant mosquitoes (3). The recent achievements in malaria control and necessity to contain artemisinin resistance have stimulated malaria elimination initiatives that require a thorough understanding of the biology and epidemiology of malaria transmission and alternative transmission-reducing interventions (4).

P. falciparum transmission to mosquitoes is initiated when a small subset of asexually replicating blood stage parasites produce sexual progeny, or gametocytes. Gametocytes develop in human red blood cells (RBC) along 5 morphological transitions (Stage I-V); stage I-IV development takes place predominantly in the extravascular niche of the bone marrow and spleen (5-7). Mature Stage V gametocytes are released into the peripheral blood circulation where they may be ingested by a blood-feeding mosquito upon which they egress from RBCs as activated gametes and fuse and form motile zygotes. Further sporogonic development renders the mosquito infectious to humans. Several sexual stage proteins have been identified that have no function in gametocyte development but are essential for gamete fertilization (e.g. Pfs48/45 and Pfs230) or post-fertilization development in the mosquito (e.g. Pfs25, Pfs28) (8).

There is currently incomplete evidence for immune responses that affect gametocyte formation, maturation or circulation time (9). Several field studies suggested mature gametocyte clearance after repeated malaria exposure (10-13) and antibody responses against uncharacterized targets on mature gametocyte-infected red blood cells (giRBCs) have been associated with lower gametocyte densities (12, 14). Another field study identified antibodies that bound the surface of stage II-V giRBCs and distorted early gametocyte morphology and maturation (15). Depending on which stage(s) they target, anti-gametocyte immune responses could be involved in blocking extravascular adhesion of immature giRBCs and/or clearance of circulating mature giRBCs, in a manner similar to antibodies against the asexual antigen PfEMP1. PfEMP1 is an immunodominant antigen on the surface of RBCs infected with asexual

parasites (aiRBC); anti-PfEMP1 antibodies have an established role in immune clearance by inhibiting vascular adhesion and by opsonizing aiRBCs for phagocytic clearance (16, 17). aiRBC surface antigens other than PfEMP1 exist (18), and are associated with phagocytosis and cytotoxicity (19). The ligands involved in giRBC adherence may be different from those involved in endothelial binding of aiRBCs; giRBCs are localized to an extravascular compartment (5, 7), show limited binding to human endothelial cell lines and harbor minimal PfEMP1 on their surface (20). Whilst no specific giRBC ligand has been identified, one tenth of the early gametocyte proteome consists of putatively exported antigens called *P. falciparum* gametocyte-exported proteins (PfGEXPs) (21).

Hypothesizing that developing gametocytes could be targets of antibody responses in the human host, we performed a systematic characterization of gametocyte stage-specific immune recognition and clearance. We demonstrate naturally acquired human immune responses targeting immature (stage I-III) but not more mature stage V giRBCs. Experiments using whole cells and surface-intact and surface-depleted membrane fractions of diverse parasite strains provide evidence for giRBC surface antigens, most of them shared with aiRBCs. We further demonstrate that natural immunity to shared iRBCs correlates with reduced asexual and gametocyte burden and that a subset of the target antigens shows minimal sequence diversity.

Results

Human immune responses recognize secreted gametocyte proteins

We first probed a *Plasmodium falciparum* peptide array enriched for proteins expressed in the gametocyte and gamete stages (22) with human plasma samples from 579 asymptotically infected individuals from Cameroon, Burkina Faso, and the Gambia (22)(table S1) to examine natural immunity. Proteins were clustered based on their stage-specific abundance in blood and mosquito stages in proteomics studies (21, 23, 24) and by cellular localization; localization was divided into those proteins that are parasite internal (internal/unknown localization) or secreted onto the merozoite or gamete surface or into the host cell in intra-erythrocytic stages (secreted)(fig. 1A-B, see also table S2). Five stage-specific clusters (gametocyte

specific or shared with asexual stages) were enriched in secreted antigens (**Figure 1A**), and secreted antigens showed higher antibody responses compared to internal antigens for shared, gametocyte-specific ($p\text{-value} = 8.86 \times 10^{-288}$) and gametocyte/mosquito stage proteins ($p\text{-value} = 4.31 \times 10^{-119}$)(**fig. 1C**). Responses to shared secreted proteins increased with age while responses to secreted gametocyte or mosquito stage proteins or to parasite internal proteins did not. Correlations were highly significant for a total of 121 individual peptides (adjusted $p\text{-value} < 0.05$, **table S3**). Although responses to numerous protein fragments showed progressive increases with age (**fig. S1A**), responses to other antigens, including PTP6 (25) and GEXP08, reached a plateau in the 12-30 year old group (**fig. S1B**). These results indicate that humoral responses to secreted parasite antigens (shared and gametocyte-specific) are correlated with cumulative exposure to malaria.

Immune responses target the immature but not the mature giRBC surface

Detection of immune responses against secreted gametocyte proteins prompted us to directly examine immune recognition of giRBC surface antigens among an independent population. In a cross-sectional study, we collected plasma samples from 244 individuals with suspected malaria from southern Malawi (see Materials and Methods and **table 1**). A subset of rapid diagnostic test (RDT)+ samples and an RDT-control (representative of the entire Malawian study population in terms of age and sex distribution) was incubated with *P. falciparum* NF54 stage II/III giRBC, stage V giRBCs, or activated gametes. Surface reactivity was measured by comparing the percentage of IgG-positive cells between incubations with Malawian and naïve control sera (see **fig. S2 and S3**). To differentiate non-activated gametocytes (i.e., intact giRBCs) from activated ones (i.e., free gametes), stage V incubations were co-stained with antibodies recognizing the gametocyte/gamete surface antigen Pfs48/45 (which becomes accessible upon RBC rupture and giRBC activation) and the RBC surface antigen Glycophorin C. Highest surface reactivity was found for gametes (mean 25.80% recognized cells), with substantial reactivity also observed for stage II/III (mean 6.22%) but not for stage V giRBCs (**fig. 2A**). The relatively low percentage of giRBCs recognized suggests low abundance, accessibility and/or immunogenicity of

putative antigen targets. Of the Malawian plasma samples tested, 75.00% (n/N=18/24) and 95.83% (n/N=23/24) recognized stage II/III giRBCs or gametes respectively, whereas no samples were positive for stage V recognition (**fig. 2B**).

We further investigated antibody specificity to immature giRBC surface antigens as compared to aiRBCs using a transgenic version of the Ghanaian *P. falciparum* parasite Pf2004 (26, 27), Pf2004_164/TdTom. This parasite expresses the TdTomato reporter under the control of the *PF10_0164* promoter (28) that allows detection by fluorescence microscopy and flow cytometry of gametocytes of all stages except the first 30 hours of development (**fig. 2C**). Among 244 Malawian plasma samples, the strongest responses to aiRBCs correlated with the strongest responses to giRBCs, whereas 14 samples were uniquely positive for giRBCs (**fig. 2D-E**). No differences between RDT+ and RDT- individuals in antibody responses for any antigen class was observed (**fig. S4A**). When we repeated our surface recognition experiments with the 3D7 reference strain (a clone of NF54 used in **fig. 2A**, potentially expressing different surface proteins than Pf2004), we observed lower surface antigen expression and lower non-specific IgG labeling from naïve serum compared to Pf2004 (**fig. S4B-C**). These strain disparities are consistent with previous work observing differential reactivity of Kenyan plasma samples to parasite strains of different genetic origins (18). Surface protein removal with trypsin/chymotrypsin revealed that both specific and non-specific binding of IgG involved antigens on the surface of aiRBCs and giRBCs (**fig. S4D-E**). Further experiments using the same patient sera and naïve controls revealed no IgM binding above background and therefore excluded IgM binding as an explanation for the observed non-specific surface recognition (**fig. S5**). These data provide strong evidence for IgG-targeted antigens that are shared between asexual and gametocyte stages.

The prevalence (number of samples with substantial aiRBC and/or giRBC recognition) and magnitude (median fluorescence intensity) of iRBC reactivity was significantly higher for adults compared to children (**fig. 2F**). The increased aiRBC reactivity with age (top panel) corroborates the well-characterized pattern of increasing breadth of antibody response to asexual parasites with cumulative exposure (29-31). The slower age-dependent increase for giRBC responses (bottom panel) may reflect the

lower abundance of immature gametocytes and suggests that giRBC responses differ from those against gametocyte/gamete antigens Pfs48/45 and Pfs230 that appear short-lived (22, 32, 33). We then probed a subset of the Malawian plasma samples (representing a range of reactivity by flow cytometry) on the peptide array to identify recognized targets. Recognition of the giRBC surface by flow cytometry was correlated with mean array responses for shared asexual-gametocyte and gametocyte-specific secreted antigens (**fig. 2G**) but not internal proteins. Individuals recognizing giRBCs by flow cytometry had significantly higher reactivity (p -value <0.05) to a subset of 22 protein fragments (including 4 shared and 13 gametocyte-specific) compared to individuals with minimal reactivity to giRBCs (**table S4** and **fig. S1C**). Altogether these data demonstrate that plasma samples recognizing both aiRBCs and giRBCs show the highest magnitude in reactivity, and this signal is driven by antibody responses against secreted antigens across all age groups.

Antigens on the giRBC surface are predominantly shared with aiRBC

TdTomato fluorescence increases with later stage gametocytes (**Figure 3A**) and microscopy and flow experiments indicated that “weak TdTomato+” corresponded to stage I/II gametocytes and “strong TdTomato+” to stage II/III gametocytes. Three lines of evidence suggest that giRBC surface reactivity is specific for stage I/II gametocytes: i) a higher percentage of stage I/II, weak TdTomato signal consistently corresponded to a higher percentage of cells staining positive for the surface (**Figure 3B**); ii) the intensity of IgG staining correlated with the percentage of weak TdTomato positive cells (**Figure 3C**); iii) microscopy confirmed significantly higher percentages of surface labeling of aiRBCs and stage I/II giRBCs compared to stage II/III giRBCs (**Figure 3D**). These results demonstrate that giRBC reactivity is highest in early stage gametocytes (stage I/II) and decreases during gametocyte development.

To identify the target giRBC surface antigens, we probed aiRBC and stage I-III giRBC membranes +/- treatment with trypsin/chymotrypsin (hereafter referred to as +trypsin and -trypsin samples) with Malawian plasma samples by Western blot. By comparing differential bands between surface-intact (-trypsin) and surface-depleted (+trypsin) samples, we identified both shared (aiRBC-giRBC) and giRBC-

specific trypsin-sensitive protein bands (**Figure 4A**), demonstrating the presence of immunogenic antigens on the giRBC surface. Next, we performed mass spectrometry-based proteomics of stage I-III giRBC membrane samples and assessed reactivity of sera from mice immunized with the same giRBC membrane samples. These results were combined with the proteins recognized by individuals with giRBC reactivity by flow cytometry in experiments described above to form an initial list of potential giRBC surface antigens.

In the first approach, we performed whole lane in-gel digestion with 3 biological replicates of +trypsin vs. -trypsin giRBC membranes and identified differentially enriched protein bands between the two conditions by mass spectrometry (**fig 4B; table S5**). Overall, 72.20% of proteins identified in -trypsin samples were shared between all 3 replicates and 92.21% of proteins were identified unequivocally in at least 2 of the 3 replicates. Out of all 235 proteins that were >1.25x enriched in the -trypsin sample (**table S5**), a subset of 30 (12.77%) secreted proteins were considered putative surface antigen candidates. Secreted proteins were defined by the presence of at least one transmembrane domain (TM, including the N-terminal signal sequence) and either known localization to membrane/surface or host cell or unknown localization. Within this set of 30 candidates, 28 (93.33%; 11.91% of total candidate list) showed evidence for export into the host cell based on predicted PEXEL motif (21 proteins) or PEXEL/HT negative exported protein (PNEP) annotation (7 proteins) and 23 were expressed in both asexual and gametocyte stages. Importantly, this candidate list includes several previously identified secreted antigens such as multiple Plasmodium helical interspersed subtelomeric (PHIST) family proteins (21, 34, 35), PIESP2 (35-37), and GEXP02 (21, 38).

In a complementary antigen-discovery approach, we immunized mice with the same surface-intact (-trypsin) or surface-depleted (+trypsin) giRBC membranes used for proteomics and probed sera on our gametocyte-enriched protein array. Several bands on Western blot were present only in experiments using sera from mice immunized with surface-intact giRBC membranes, and were reduced in intensity when surface-depleted membranes were probed with these sera compared to surface-intact membranes (**fig. 4C**). Sera from all mice showed similar responses to parasite-internal peptides on the array, but sera from

mice immunized with –trypsin preparations showed significantly higher responses to secreted proteins compared to mice immunized with +trypsin preparations (p -value=0.04315)(**fig. 4D**). Due to lower background using mouse sera compared to human sera, many normalized mean response values were negative; however, the significant differential responses were consistent with observed reduced band intensity after trypsin treatment by Western blot (**fig. 4C**) and with the same array probed with human plasma samples described earlier. Consistent with our previous results using the peptide array, 16 individual protein fragments elicited significantly higher differential responses with sera from mice immunized with surface-intact membranes (**fig. 4E, table S6**). Notably, GEXP07 and GEXP10, two proteins on the iRBC surface that can bind to the chemokine CX3CL1 (37) were recognized both by sera from mice immunized with intact and surface-depleted membranes (**fig. 4F**), suggesting that their ectodomain is trypsin insensitive.

In total, we identified an overlapping set of 68 initial candidate giRBC surface antigens: 22 proteins with significantly correlated array vs. flow cytometry responses (**table S4**), 30 proteins from mass spectrometry-based proteomics (**table S5**), and 16 proteins eliciting significantly higher responses from sera from mice immunized with surface-intact (compared to surface-depleted) giRBC membranes (**table S6**). This list was then filtered based on detection by gametocyte surface proteomics and presence of at least one TM; subsequently any proteins with confirmed localization within the parasite or parasitophorous vacuole or Maurer's clefts were removed. The remaining 30 proteins were therefore deemed potential giRBC surface antigens (**table S7**): 26 were identified by surface proteomics, 3 by the parallel mouse immune profiling experiment and 1 hit was identified only by correlating protein array responses and surface reactivity of patient plasma samples. Of the 30 candidate antigens, 26 (86.7%) showed evidence of export into the host cell based on the presence of a PEXEL (23 proteins) or PNEP (3 proteins) motif, and the majority of the identified proteins (23: 76.7%) were expressed both in asexual and gametocyte stages (i.e., shared expression profile). Importantly, there is independent evidence for localization at the iRBC periphery and/or surface for 12 out of these 30 candidates from previous studies (**Supplementary table S7**), further supporting our data.

Validation of giRBC antigen surface localization

From the 30 proteins, we selected 9 for experimental validation of surface expression using antibodies against peptides (PF3D7_0402000, PF3D7_0702500, PF3D7_0936800, PTP5, PTP6, GEXP02 and GEXP10; GEXP07 and GEXP10 (37)), or recombinant protein (PF3D7_0532400 (39)) (**table S8**) in Western blots (**fig. 5A**), flow cytometry (**fig. 5B**), and live immunofluorescence assays (**fig. 5B-C**). In addition, we performed IFAs using fixed, permeabilized cells to determine the cellular distribution of the candidate proteins (**fig. 5D**). We obtained a band of the expected size by Western blot, and candidate antigens showed variable degrees of trypsin sensitivity (**fig. 5A and fig. S6**). All antibodies except PTP6, which did not detect giRBCs, were then tested by flow cytometry (**fig. 5B**) and immunofluorescence microscopy (**fig. 5B-C**) using live Pf2004/164TdTomato parasites. By flow cytometry, all antibodies, except those against GEXP10 and GEXP07, showed significantly reduced recognition of surface-depleted asexual stages and early gametocytes although cell binding was low for some antibodies (**fig. 5B, right panel**). The overall percentage of cells labeled, as well as the magnitude of decreased labeling after trypsin treatment, were higher by live IFA **fig. 5B, left panel; fig. 5C**) compared to flow cytometry. Again, GEXP10 and GEXP07 appeared insensitive to trypsin treatment in these assays. Apart from trypsin sensitivity we quantified the proportion of surface-labeled aiRBCs and giRBCs, the fluorescence intensity of surface labeling, and the average percentage of surface coverage among labeled cells by live microscopy (**fig. 5C, left panel**). Whereas GEXP10 and GEXP07 showed high levels for all 3 measurements, other antibodies had high values for one or two parameters (**fig. 5C**). Automatic independent clustering by all 3 measurements simultaneously confirmed 6 candidates - PTP5, GEXP02, PF3D7_0936800, GEXP07, GEXP10 and PF3D7_0702500. In contrast, the two candidates with major expression in asexual stages and minimal expression in gametocytes based on our proteomic clustering (PF3D7_0402000, PF3D7_0532400) showed the lowest levels of giRBC surface staining by live microscopy. Finally, immunofluorescence microscopy using fixed and permeabilized cells confirmed significant labeling at the iRBC periphery, and in addition, co-labeling with the Maurer's Cleft marker

SBP1, for 3 of these candidates across asexual and immature gametocyte stages (**fig. 5D**). Antibodies against all three candidates showed markedly weaker labeling in gametocytes compared to asexual parasites. Altogether, analysis of a subset of candidates using peptide antibodies validated our analysis pipeline and confirmed six proteins as giRBC surface antigens.

A subset of secreted parasite antigens shows minimal sequence diversity and elicits responses that are correlated with reduced gametocyte burden

To determine the extent of sequence polymorphisms amongst the antigens analyzed in this study, we measured signatures of selection in the encoding genes from clinical isolates collected from two patient populations in Senegal and Malawi (**table S9**). Analysis of nonsynonymous pairwise nucleotide diversity (π_{NS}) demonstrated significantly elevated levels of genetic diversity in genes encoding secreted compared to internal antigens across all stages (**fig. 6A and S7A**; Mann-Whitney U test, $p = 8 \times 10^{-13}$ (Senegal), $p = 3.7 \times 10^{-11}$ (Malawi)). Genes with Tajima's D values above the genome-wide 95th percentile ($D > -0.343$), indicating balancing selection, were also enriched in secreted relative to internal antigens (**fig. S7B**; Fisher's Exact Test, $p = 0.0153$ (Senegal), $p = 0.00660$ (Malawi)). These data support the hypothesis that acquired immunity drives genetic diversity in genes encoding secreted *P. falciparum* blood stage antigens (both shared and gametocyte-specific). Indeed, we measured a positive correlation between immune responses against secreted antigens and the levels of π_{NS} of the encoding genes (Pearson's correlation; $r = 0.221$, $p = 0.000141$ (Senegal); $r = 0.199$, $p = 0.000623$ (Malawi)). Levels of π_{NS} were significantly increased at mean responses greater than 0.5 across secreted antigens, suggesting a threshold effect inducing positive selection through antibody-mediated immunity (**fig. 6A**; Mann-Whitney U test, $p = 0.0265$ (Senegal), $p = 0.0441$ (Malawi)). We also quantified genetic differentiation between the two geographically separated parasite populations in Malawi and Senegal using the fixation index (F_{ST}). This analysis demonstrated that genes encoding secreted antigens show significantly higher F_{ST} indices (Mann-Whitney U test, $p = 2.3 \times 10^{-5}$), and that the majority of genes had high corresponding indices ($F_{ST} > 0.1$) (**fig. 6B**; Mann-Whitney U test, $p = 2.3 \times 10^{-5}$). Amongst our 30 candidate antigens, 9 showed minimal

levels of nucleotide diversity across parasite populations in Malawi and Senegal (**fig. S7C**, and **table S9**). Seven antigens, including the validated surface antigens GEXP07 and PTP5, show both minimal levels of nucleotide diversity across parasite populations and low levels of population divergence between populations (**fig. S7C**, and **table S9**). Altogether, genetic analysis demonstrates that genes encoding secreted antigens show significantly higher signatures of selection compared to internal antigens whilst a subset of eight antigens show minimal levels of genetic diversity and may thus elicit strain-transcending immunity (**table 2**).

It is currently unknown whether antibodies recognizing shared or gametocyte-specific surface antigens may inhibit giRBC binding/sequestration and/or increase phagocytosis efficiency by opsonization - as implicated in responses to PfEMP1 (18, 40, 41) and merozoite antigens (42, 43). To directly test this hypothesis, we opsonized iRBCs with the same Malawian plasma samples used for iRBC surface labeling and determined the level of iRBC phagocytosis by THP-1 cells (18). Significant levels of iRBC phagocytosis were detected (**fig. 6C**), and the magnitude of surface reactivity was significantly correlated with induction of phagocytosis both for aiRBCs and giRBCs (**fig. 6D**). Altogether these data demonstrate existence of functional antibodies targeting both aiRBCs and giRBCs, and provide evidence for antibody-mediated clearance of giRBCs. In support of these functional assays, the intensity of recognition of shared secreted antigens by plasma samples from individuals in Cameroon, Burkina Faso, or the Gambia was overall negatively associated with the gametocyte fraction in these individuals (quantified by coefficients of regressing antigen response on logit-transformed gametocyte fraction). In contrast, normalized recognition of asexual antigens was overall negatively associated with asexual stage and gametocyte load (also quantified by regression coefficients, antigen response on log-transformed asexual/gametocyte load), whereas normalized recognition of gametocyte-specific antigens did not show any negative association (**fig. 6E**). Furthermore, the proportion of total parasites that were gametocytes was negatively associated with breadth of response to the 76 fragments representing the 31 candidate surface antigens on the peptide array (coefficient, -0.002 (95% CI -0.004/-0.0004), $p=0.019$). Importantly, responses to a total of 12 candidate surface antigens, including three of our final candidates (Table 2)

showed significant ($p<0.05$) negative correlation between immune response and both peripheral gametocyte and asexual stage load (**fig. 6F and S8, and table S10**). These data support the phagocytosis data and suggest that iRBC immunity may be able to simultaneously reduce total parasite burden and gametocyte burden.

Discussion

In this study, we systematically addressed immune recognition of antigens on the surface of giRBCs and provide evidence for the identity of these proteins. Our combination of a flow cytometry assay using distinct gametocyte stages, immune profiling by protein microarray, 3 parallel methods of antigen discovery, and a functional assay to quantify antibody-mediated iRBC phagocytosis, provides evidence for naturally acquired antibodies recognizing shared asexual/gametocyte and gametocyte-specific antigens on the surface of immature giRBCs.

Two previous studies reported immune recognition of mature giRBCs (12, 14) but did not specifically control for gametocyte activation. We regularly observed glycophorin-negative gametocyte populations where the giRBC membrane was lost due to activation or permeabilization. It is conceivable that earlier studies have similarly experienced a loss in RBC integrity and may thus have detected antibodies against gamete proteins, that are common in endemic populations (22), instead of mature giRBC responses. Less stringent methods of giRBC purification also could have hindered detection of responses targeting the most immature stages. When we carefully prevented activation by using a compound that prevents gamete egress (44), and confirmed the intact RBC membrane by counterstains (the gamete surface antigen Pfs48/45 and the RBC surface antigen Glycophorin C), we did not detect significant recognition of stage V giRBC. In addition, we observed strong reactivity to stage I/II gametocytes but negligible reactivity to stage V gametocytes in our highly synchronous TdTomato transgenic parasite line (45). Our data demonstrate that plasma from naturally exposed individuals strongly recognizes early stage I/II giRBCs and aiRBCs; the majority of immunogenic giRBC antigens in our study are also expressed in asexual stage parasites. These observations have potential implications for

our understanding of parasite biology. Asexual and early gametocyte stages of *P. falciparum*, *P. vivax*, and *P. berghei* are abundantly present in the bone marrow parenchyma (5, 7, 46, 47), suggesting environmental characteristics supporting both gametocyte development and a genuine asexual replication cycle. An independent study recently confirmed that both bone marrow and spleen represent major reservoirs for parasite development in rodent malaria (48). We hypothesize that shared antigens present on aiRBC and giRBC surfaces are involved in cellular interactions in the bone marrow parenchyma and critical for the maturation of both asexual and gametocyte stages. In such a model, the aiRBC surface serves the dual purpose of vascular adherence and extravascular binding, while the giRBC surface is optimized for extravascular binding only. Indeed, recent work demonstrated trypsin-sensitive binding of aiRBCs and immature but not mature giRBCs to human bone marrow mesenchymal stromal cells (49). Interestingly, two antigens we identified on the giRBC and aiRBC surface, GEXP07 and GEXP10, were recently described as aiRBC surface proteins that bind the chemokine CX3CL1 (37). As expression of this chemokine on bone marrow stromal cells is involved in homing and retention of monocytes (50), it is tempting to speculate that GEXP07 and GEXP10 are involved in such interactions between iRBCs and other cell types. It remains to be determined why human IgG levels recognizing giRBC antigens are generally lower compared to aiRBCs and why recognition is restricted to young gametocyte stages, despite their continued presence in the extravascular niche until maturity. Although we only examined stage I/II, III and V gametocytes, and not the intermediate stage IV, our data suggest reduced antigen expression on the giRBC surface over the course of gametocyte development, the mechanism of which could include a combination of membrane remodeling, protease activity, or release via extracellular vesicles. As the molecular mechanisms of the bone marrow sequestration process become further elucidated, the ability and function of natural antibodies to access this compartment in meaningful concentrations and effectively target parasites in this niche is likely to also be revealed.

Our data reveal a positive correlation between antibody-mediated immunity and genetic diversity in secreted parasite antigens. Nevertheless, we identified a small set of immunogenic candidate antigens with minimal genetic diversity within and between populations, suggesting that they may induce strain-

transcendent immunity. Our plasma samples were from cross-sectional surveys in asymptomatic populations. Whilst this makes it unlikely that inflammation or acute disease have influenced the results, our sampling approach means we were lacking details on gametocyte commitment and maturation, and were thus unable to test causality between antibody responses and parasite and gametocyte dynamics. We observed that the proportion of the total parasite biomass that is gametocyte (indicating what fraction of parasites successfully develops into circulating mature gametocytes (51)) was reduced in infections of individuals who responded to peptides mapping to shared asexual/gametocyte antigens. The negative associations between responses to asexual secreted antigens and asexual parasite load suggest a specific role for these proteins in reducing asexual parasite burden, in addition to the established contribution of anti-PfEMP1 antibodies (18, 19). Importantly a total of 12 candidate antigens, including 3 of our 8 top candidates with low sequence diversity, showed negative correlations between antibody titer and both asexual and gametocyte load, suggesting an association with reduced parasite growth and gametocyte maturation or clearance. This possible phenotype of the detected antibody responses is supported by our finding that plasma samples with increased aiRBC and giRBC surface recognition demonstrate increased phagocytosis of aiRBC and giRBC by THP-1 cells. This phenotype and the identification of a small set of target immunogenic antigens present on the giRBC surface with low sequence diversity, provides a rationale for a novel transmission blocking vaccine strategy that may interfere with gametocyte maturation. Such a vaccine approach would reduce the number of gametocytes in the circulation and hence transmission potential.

Altogether, we provide compelling evidence for natural immune responses targeting young gametocytes and their antibody-mediated immune clearance. We identify a small set of 8 candidate antigens that are i) expressed in gametocytes (7 of them are also expressed in asexual stages), ii) elicit natural antibody responses and iii) display low sequence diversity.

Materials and Methods

Study design

For the Malawi study, samples were collected over 4 weeks in July/August 2013. Two weeks were spent in Chikhwawa, as this region had higher malaria transmission during this time of year and one week each in Ndirande and Thyolo. All individuals receiving an RDT at the clinic were referred to our study and samples were taken from all of those individuals who consented to the study. The end of data collection was not determined by any factor other than the end of the defined sample collection period. Samples from two individuals who withdrew their consent after participation were discarded; all other samples were shipped to the US for further experiments. We aimed to detect natural antibody responses among the study participants that recognize giRBCs and then to determine the targets of these antibody responses. To examine antibody binding to the giRBC surface, we used a surface reactivity flow cytometry assay, immunofluorescence microscopy and a protein array enriched for proteins expressed during gametocyte stages. In these experiments, samples were identified only by number and patient age and corresponding clinical data was unblinded only after experiments finished. Three technical replicates were used for all samples and two biological replicates were performed for a subset of samples. In cases where the result from one technical replicate was of a different magnitude than the other 2 replicates, this value was removed. To determine the identity of antigens targeted by the identified antibodies, we used mass spectrometry and immunization of mice with giRBC membranes, each using 3 biological replicates for preparation of giRBC membranes. Surface expression of candidate antigens was validated by Western blot, flow cytometry, and immunofluorescence microscopy. Functional activity was assessed using a THP-1 cell phagocytosis assay. Sequence diversity was assessed using standard methods (nonsynonymous pairwise nucleotide diversity, balancing selection measured by Tajima's D, genetic differentiation measured by the fixation index).

Statistical analysis

The appropriate statistical test for each experiment was determined based on the type of data being compared. FDR corrections were performed for all analyses involving multiple comparisons and p -values <0.05 were considered significant. Simple univariate linear regressions were performed for examining the

correlation between levels of IgG responses against individual fragments on the protein array and covariates including (ordinally categorized) age, burden, and iRBC recognition by flow cytometry. *P*-values across fragments were corrected with Bonferroni method. Pairwise, two-sided student t-tests were used to test for difference in mean IgG response against proteins across stages. Linear regressions were used to test for associations between IgG response against fragments and parasite load, gametocyte load and gametocyte fraction, with adjustment for age by including age groups as covariates. The regression t-statistics (estimated coefficients / standard error) of internal and secreted protein fragments are compared by two-sided Mann-Whitney U test. The association of gametocyte fraction and breadth of response (number of proteins seropositive) was conducted on gametocyte positive individuals for whom asexual and gametocyte stages had been quantified. Analysis on breadth and fraction on continuous scales was performed with linear regression, adjusting for gametocyte density. Analysis with breadth as a binary variable was performed with logistic regression, adjusting for gametocyte density. Throughout the manuscript significant *p*-values are reported either as is or with the corresponding alpha-level (all < 0.05).

Supplementary materials

Materials and Methods

Fig. S1. Correlations between age and reactivity by peptide array or between reactivity by peptide array and reactivity by flow cytometry.

Fig. S2. Schematic of gating strategy for measuring giRBC surface reactivity by flow cytometry.

Fig. S3. Activation of stage V gametocytes and the impact of protein kinase G inhibitors on activation.

Fig. S4. Stage-specific reactivity of human plasma with iRBCs by flow cytometry.

Fig. S5. Human IgM binding to iRBCs.

Fig. S6. Specificity of polyclonal antibodies against candidate antigens by Western blot.

Fig. S7. Genetic diversity and divergence of candidate antigens.

Fig. S8. Antibody correlations and protein details from 3 top candidates (**table 2**).

Table S1. Protein array details and mean responses of patient plasma samples tested on protein array.

450 Table S2. Annotation of proteins on the array.

451 Table S3. Correlations between mean responses and age.\

452 Table S4. Correlations between mean responses and giRBC surface reactivity by flow cytometry.

453 Table S5. Proteomics hits identified by LC-MS/MS.

454 Table S6. IgG responses from mice immunized with gametocyte ghosts.

455 Table S7. Candidate gametocyte antigens identified by three complementary methods (expanded from

456 table 2).

457 Table S8. Amino acid sequences for peptide antibodies generated in this study.

458 Table S9. Genetic diversity data for all genes analyzed in this study.

459 Table S10. Correlations between mean responses by array with parasite load.

460 Data file S1. Primary data

461

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Author contributions:

K.W.D., S.R., T.B. and M.M. designed the experiments in this study. K.W.D. performed flow cytometry experiments, Western blots, preparation of giRBC membranes, and immunofluorescence microscopy with help from S.R., M.M.J. and T.R. (flow cytometry with stage V gametocytes and gametes), S.K.N.B. (preparation of aiRBC membranes and design of mouse immunization studies), and M.D.N. (live immunofluorescence microscopy). S.M. and C.H. designed and executed statistical analysis of protein array data. D.T., C.U-M. and R.D. conducted mass spectrometry-based proteomics. W.J.R.S., T.B. and J.J.C. developed the protein array down-selected from a larger array originally developed by H.D. and P.F. J.J.C. and A.A.T. designed protein microarray experiments, A.A.T. produced gametocyte protein arrays, and T.Q.L. and C.L.W. generated protein array data. P.N. performed phagocytosis assays and protein clustering analysis. L.L. performed high content image analysis of iRBCs. A.M.E. and D.N. performed population diversity analysis. T.T., K.S. and M.L. helped set up the data collection in Malawi while I.M. set up the cohorts in Burkina Faso, Cameroon, and the Gambia. P.H. and P.D. provided GEXP10 and GEXP07 antibodies and helped with data interpretation. D.F.W. was involved in study design and helped with data interpretation. K.W.D., T.B. and M.M. wrote the manuscript with input from all co-authors.

Competing interests:

P.F. is inventor on patent application # US20180016299A1 submitted by University of California that covers Protein Microarray Construction.

Data and availability:

All data associated with this study are present in the paper or Supplementary Materials. Sample meta data and protein microarray data are available at <https://datadryad.org/resource/doi:10.5061/dryad.8bp05>.

Whole genome sequence data for the Senegal and Malawi samples are publicly available on the MalariaGen Pf3k website (<https://www.malariagen.net/projects/pf3k>). Scripts and example input files

used for calculating population genetics statistics are available at

https://github.com/amearly/Dantzler_et_al_Diversity_Calcs.

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Figures legends

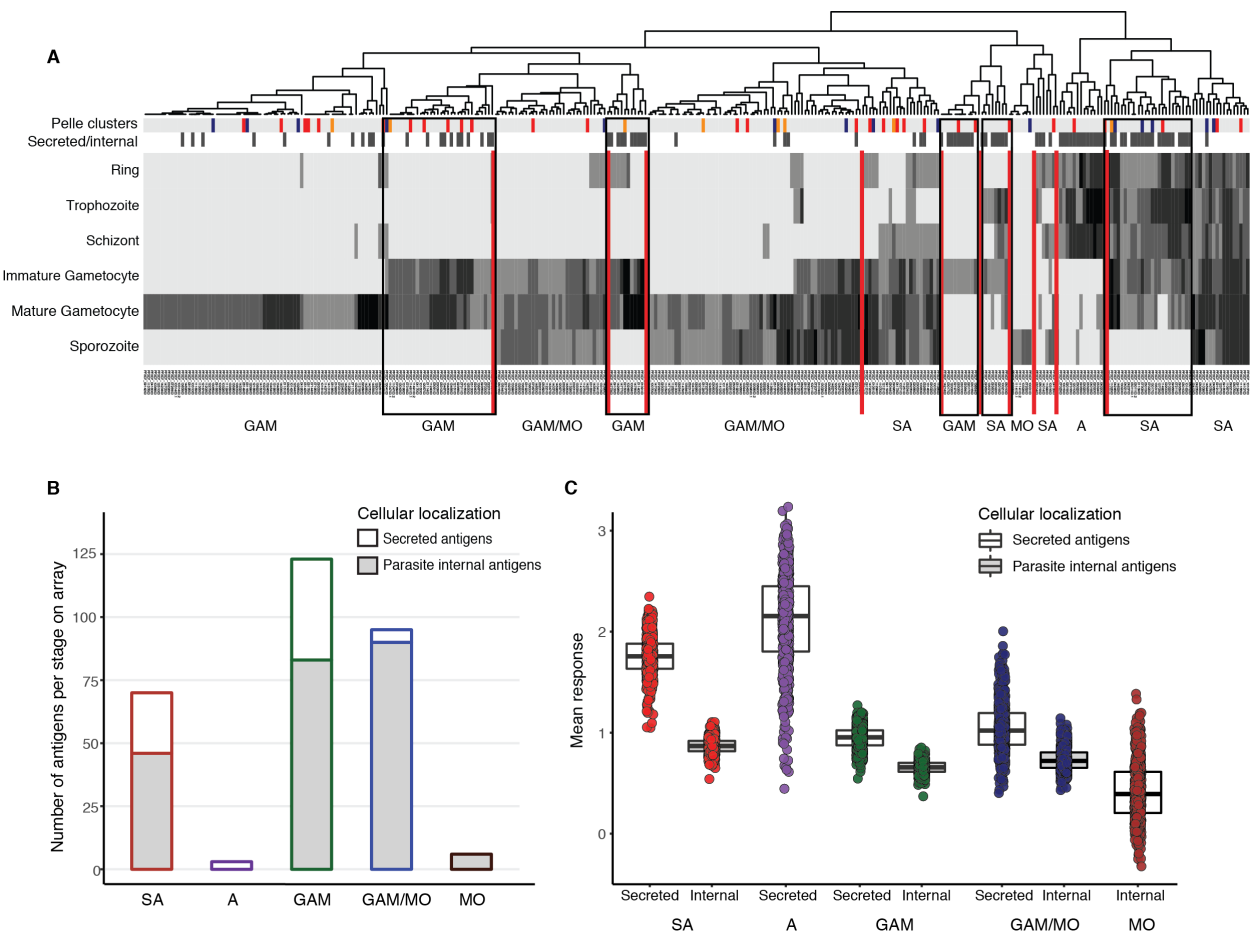


Figure 1: Human plasma samples recognize secreted asexual (aiRBC) and gametocyte (giRBC) surface antigens.

A. Heat map of 344 *P. falciparum* antigens from 3D7 genome (PlasmoDB Release 31) clustering proteins on the array by timing of protein expression (log read counts of number of peptides sequences). Additional annotations are indicated by color bars at the top of the heat map: first row indicates cluster stage annotation from (52) (orange: gametocyte rings, red: immature gametocytes, blue: mature gametocytes, grey: others), and second row indicates cellular localization (black: secreted, white: internal/unknown). Vertical red lines separate stage-specific clusters. Black boxes highlight 5 clusters of shared or gametocyte-specific secreted antigens. **B.** Distribution of 528 *P. falciparum* protein fragments on the peptide array (developed in (22)) by stage and location. The proteins were selected based on

795 expression during gametocyte stages and predicted export (details in **table S2**). **C.** Mean responses across
796 3 malaria-exposed populations are quantified by peptide array (after normalization to controls and
797 quantile normalization), stage of protein expression, and whether they are secreted or not (see **table S1**).
798 GAM=Gametocyte, GAM/MO=Gametocyte/Mosquito stages, MO=Mosquito stages, A=Asexual stages,
799 SA=Shared antigens.
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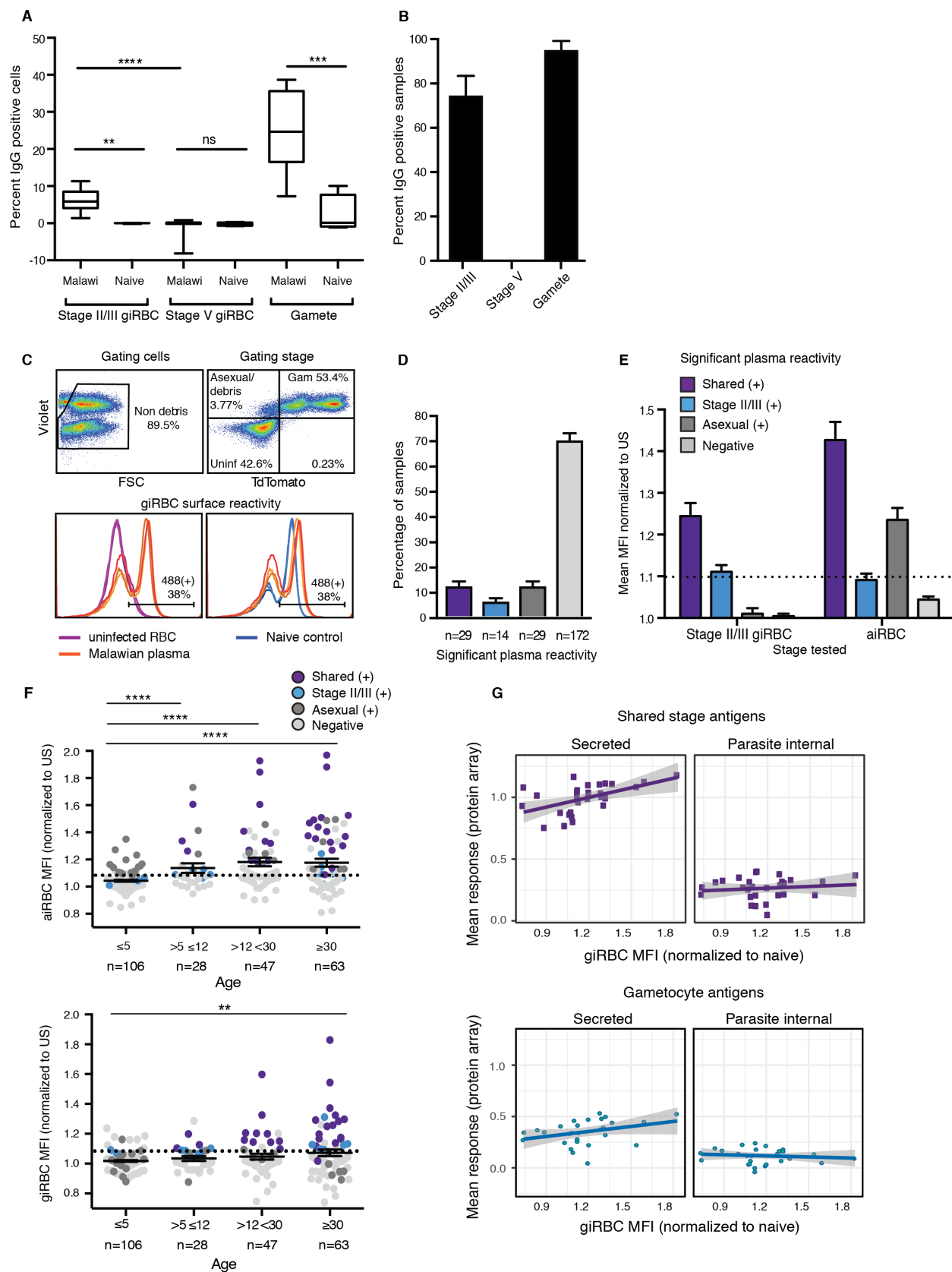


Figure 2: Immune responses target the immature but not the mature giRBC surface.

A-B. Results from a pilot flow cytometry study testing reactivity of 24 Malawian plasma samples (22 from Chikhwawa, a high transmission region, and 2 from Ndirande, a low transmission region) and 5 naïve controls against stage II/III and stage V gametocytes and gametes. Positive surface reactivity (> 3 standard deviations above mean of naïve controls) is shown both as percentage of significantly positive samples of all those tested (**A**) and percentage of positive cells among those incubated with an individual plasma sample (**B**). **C.** Schematic for gating strategy of giRBC surface detection in 244 Malawian plasma samples by flow cytometry. IgG positivity is determined using the Pf2004_164/TdTom line that allows selection of the parasite population (positive for DNA dye) and TdTomato (positive for gametocytes). Top panel: Cells are first gated for live cells and single cells by forward and side scatter (left). After debris is gated out, quadrant gates separate gametocytes (Violet⁺/TdTomato⁺), asexual/lysed cells/debris (Violet⁺/TdTomato⁻), and uninfected cells (right). Bottom panel: AlexaFluor488 surface fluorescence (human IgG-secondary antibody conjugates) is compared between uninfected cells and gametocytes (left), and between infected cells incubated with naïve controls and Malawian plasma samples (right). Technical replicates are shown as individual lines. **D-E.** Positive recognition of aiRBCs and stage II/III giRBCs (determined by t-tests comparing Malawi samples to naïve US controls using the Holm-Sidak method with $\alpha=0.05$) by 244 Malawian plasma samples is shown as prevalence (**D**) and as significant fold change in AlexaFluor488 median fluorescence compared to naïve controls (**E**). The threshold for specific positive reactivity was set to 1.1 based on the highest level of non-specific reactivity (i.e. reactivity to aiRBCs of human plasma significantly positive for stage II/III giRBC but negative for aiRBC). **F.** Correlation of human plasma recognizing aiRBCs (top panel) and giRBCs (bottom panel) by flow cytometry with age. **G.** Correlation of antigen responses by peptide array vs. surface recognition by flow cytometry. For the same set of Malawi plasma samples, normalized peptide array signal intensities were averaged across all shared stage antigens (top panel) or gametocyte-specific antigens (bottom panel). These mean responses were correlated with giRBC recognition by flow cytometry as measured by median fluorescence (AlexaFluor488) fold change compared to naïve controls. Overall, mean responses of shared

828 secreted antigens are significantly correlated with giRBC recognition ($p=0.004$), whereas the other
829 correlations are non-significant. Significance values: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p <$
830 0.0001 .

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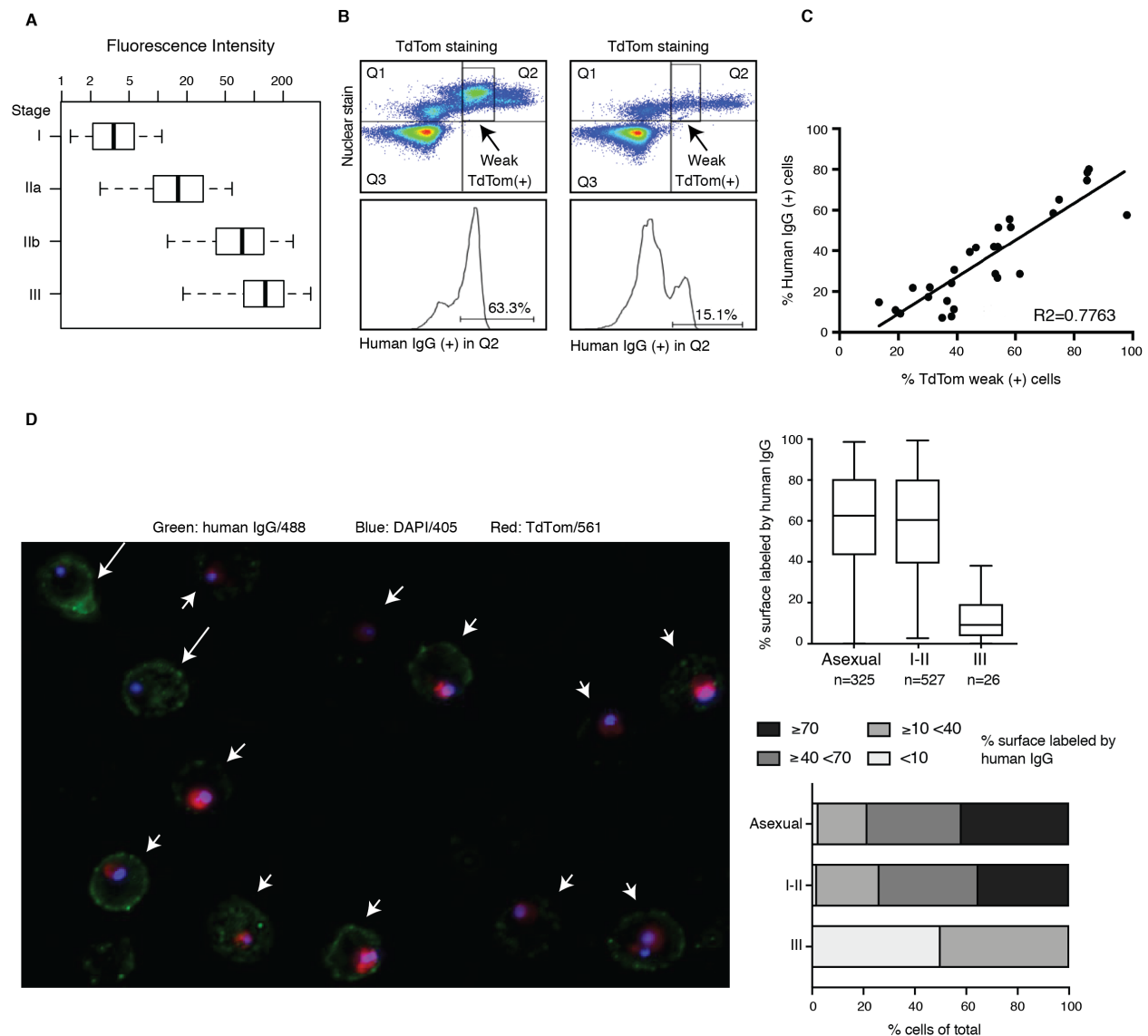


Figure 3: Human IgG selectively recognizes the early (stage I/II) giRBC surface.

A. Stage-specific expression of the TdTomato reporter in transgenic Pf2004/164TdTom parasites by flow cytometry. Reporter expression is shown by fluorescence intensity in a time course across stage I-III gametocytes. **B.** The Violet+TdTomato+ gametocyte population detected by flow cytometry can be separated into weak TdTomato+ (stage I/II gametocytes) and strong TdTomato+ (stage II/III) subgroups. **C.** Correlation between TdTom signal and human IgG based on flow cytometry data (Pearson's correlation, $p<0.0001$). **D.** Fluorescence microscopy analysis using the same antibody and reporter combination as above. Left panel: Surface labeling is present on both asexual parasites (arrows) and early

841 gametocytes (arrowheads). Right panels: High content image quantification of fluorescence microscopy
842 data, based on proportion of the cell surface that is labeled (top panel) and stratified by intensity (bottom
843 panel).

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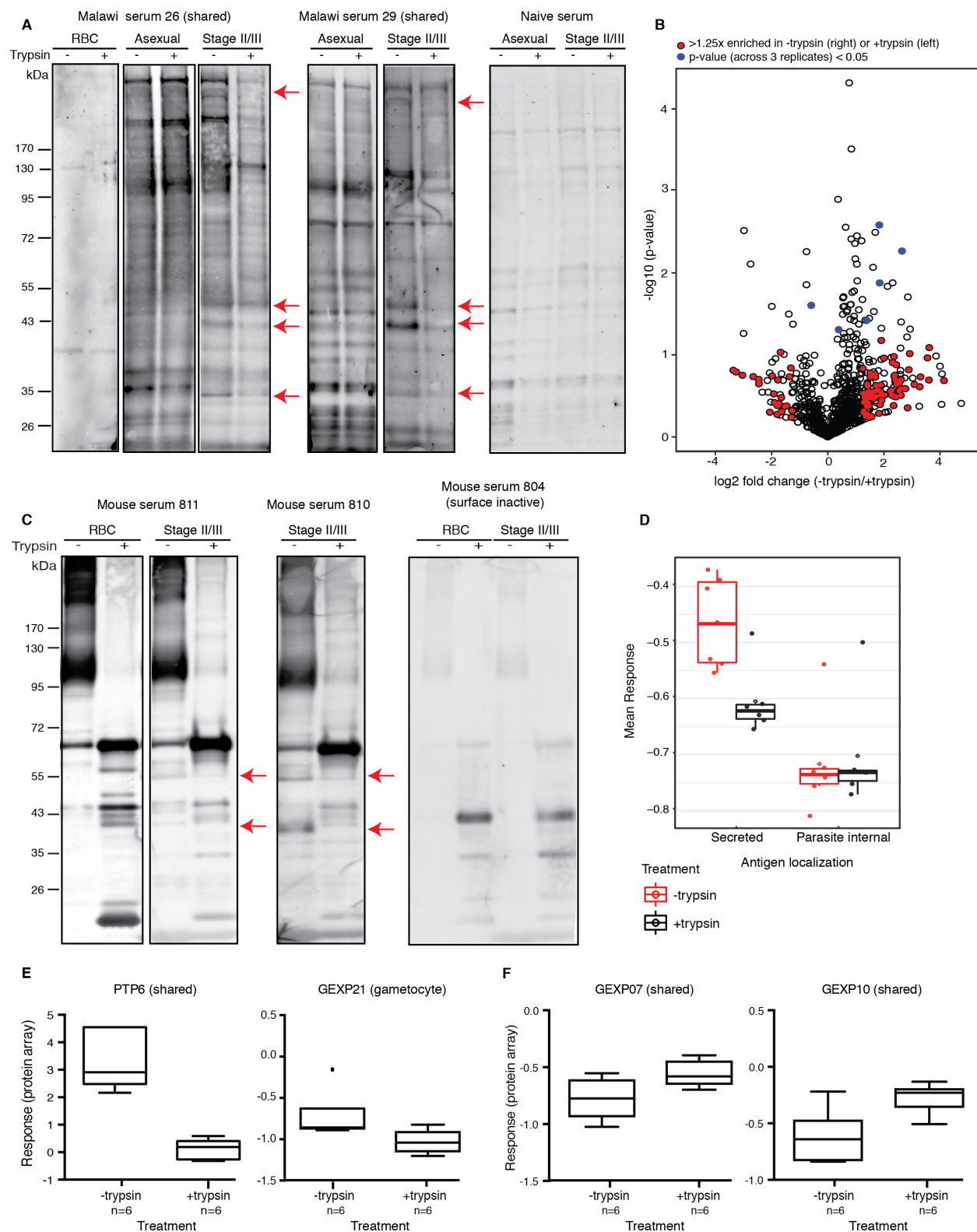


Figure 4: Immunogenic gametocyte antigens identified by 3 complementary approaches. A. Surface-depleted vs. surface-intact uninfected and infected RBC membranes were probed with Malawian plasma

848 samples and naïve US sera by Western blot. Each lane represents protein extract from 2.5×10^6 uRBC or
849 iRBC. Differential band patterns between the trypsin(+) and trypsin(-) samples are marked with red
850 arrows. **B.** Volcano plot showing human and *Plasmodium* proteins identified by comparing surface-intact
851 vs. surface-depleted giRBC membranes. X-axis represents log2 fold change of -trypsin/+trypsin and Y-
852 axis shows the T-test *p*-value ($p < 0.05$ corresponds to *p*-value 0.0004 after Benjamin-Hochberg
853 correction) of -trypsin/+ trypsin biological replicates (n=3). *Plasmodium* proteins with a log2 fold change
854 >1.25 are marked in red and significant *Plasmodium* proteins across 3 replicates are marked in blue. **C.**
855 Surface-depleted (+trypsin/chymotrypsin) vs. surface intact (-trypsin/chymotrypsin) uRBC and giRBC
856 membranes were probed with sera from mice (6 per group) immunized with surface-intact or surface-
857 depleted giRBCs by Western blot. Each lane represents protein extract from 2.5×10^6 uRBC or iRBC.
858 Differential band patterns between the trypsin(+) and trypsin(-) giRBC samples are shown in red. **D.** The
859 array described in **Figure 1** was probed with sera from mice immunized with either surface-depleted or
860 surface-intact giRBC membranes. Responses were normalized to controls and then quantile normalized.
861 **E.** PTP6 and GEXP21 differential responses between sera from mice immunized with surface-intact (-
862 trypsin) giRBC membranes and surface-depleted (+trypsin) giRBC membranes. **F.** GEXP07 and GEXP10
863 differential responses from sera from mice immunized with intact and surface-depleted membranes. See
864 **table S7** for complete data set.

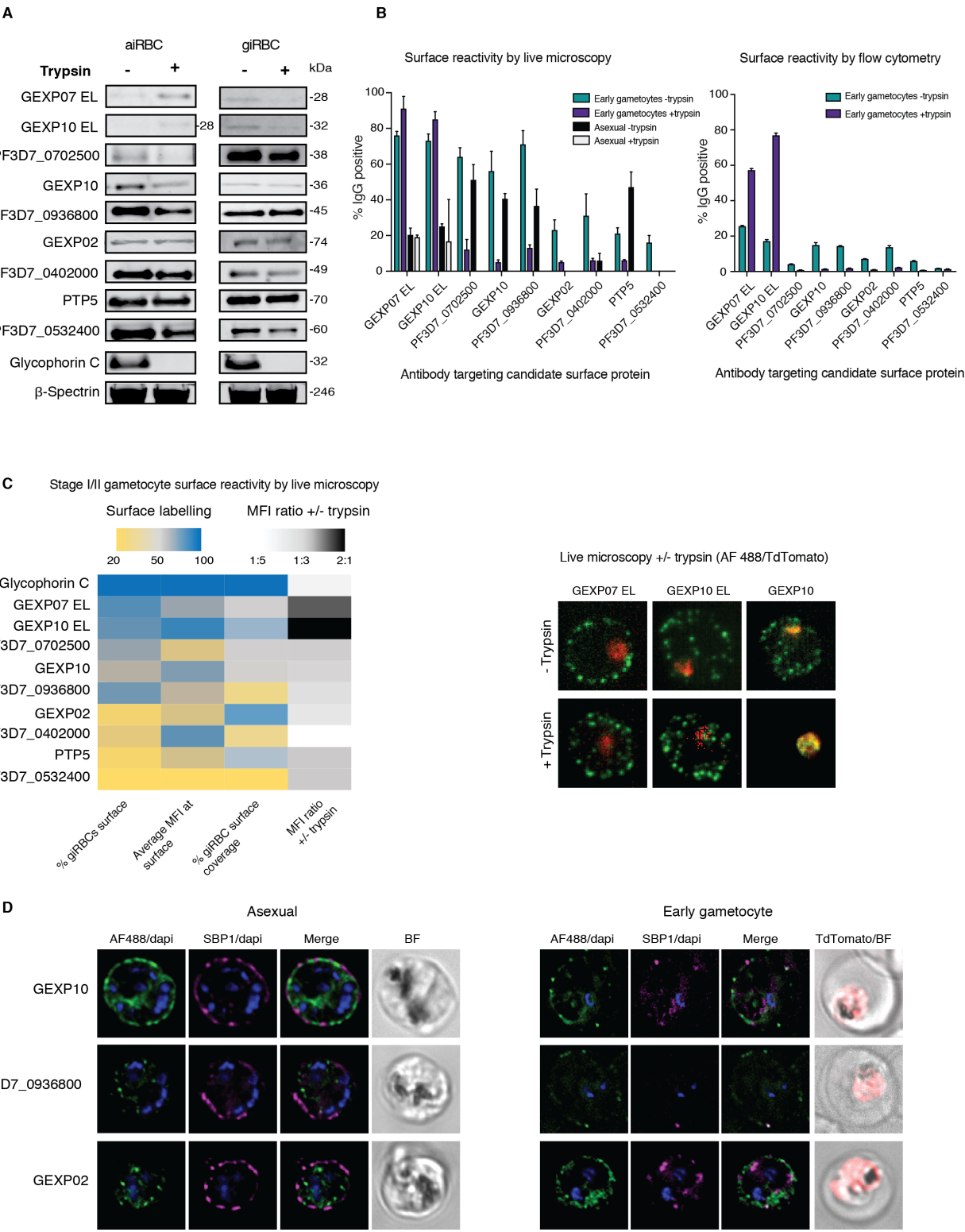


Figure 5: Six candidate antigens expressed during gametocyte stages are validated on the giRBC surface.

Previously published GEXP07 and GEXP10 antibodies (37) target the putative extracellular loops of these proteins and will be referred to as “GEXP07 EL” and “GEXP10 EL” to distinguish from our newly produced peptide antibodies targeting the same proteins. **A.** MACS purified aiRBC or giRBC membranes (+/- pre-treatment with trypsin/chymotrypsin: hereafter referred to as +/- trypsin) are probed with polyclonal antibodies targeting candidate antigens by Western blot (see full blots in **Supplemental Figure S6**). Antibodies against Glycophorin C (trypsin-sensitive, surface expressed) and β -spectrin (trypsin resistant, internally localized) are included as controls. Each lane represents protein extract from 2.5×10^6 iRBC. **B.** Reactivity of candidate antibodies to surface of MACS-purified Pf2004/164TdTomato iRBCs (+/- trypsin/chymotrypsin) was detected by live microscopy (left panel) and flow cytometry (right panel), using the same sample preparations in parallel. For live microscopy, the percentage of surface labeled aiRBCs or stage I giRBCs (weak TdTomato+) are shown for all antibodies tested. No asexual samples were tested for GEXP02 and PF3D7_0402000. For flow cytometry, cells were gated for live cells, single cells, and then uRBCs and giRBCs were gated based on Vybrant Violet and TdTomato fluorescence and surface reactivity measured using AlexaFluor488-conjugated secondary antibody. The TdTomato positive population was further split into “weak TdTomato+” (corresponding to earlier gametocytes) and “strong TdTomato+” (corresponding to later gametocytes) populations. **C.** Antibodies were clustered (automatic independent clustering) based on the imaging parameters shown in the heatmap: percentage of labeled giRBCs, average MFI at the giRBC cell surface, % giRBC surface covered, and ratio of MFI at the surface of -trypsin samples compared to +trypsin. Glycophorin C is included as a control. Live representative images of early giRBCs +/-trypsin treatment are shown for GEXP07 and GEXP10. **D.** Immunofluorescence analysis of the localization of GEXP02, GEXP10, and PF3D7_0936800 (detected with anti-peptide antibodies) in fixed, permeabilized aiRBCs and giRBCs (days 2 and 4 of the induction, corresponding to stages I-IIA and IIA-IIB, respectively). Candidate protein is shown in green, SBP1 in magenta, TdTomato in red, and nuclear staining in blue.

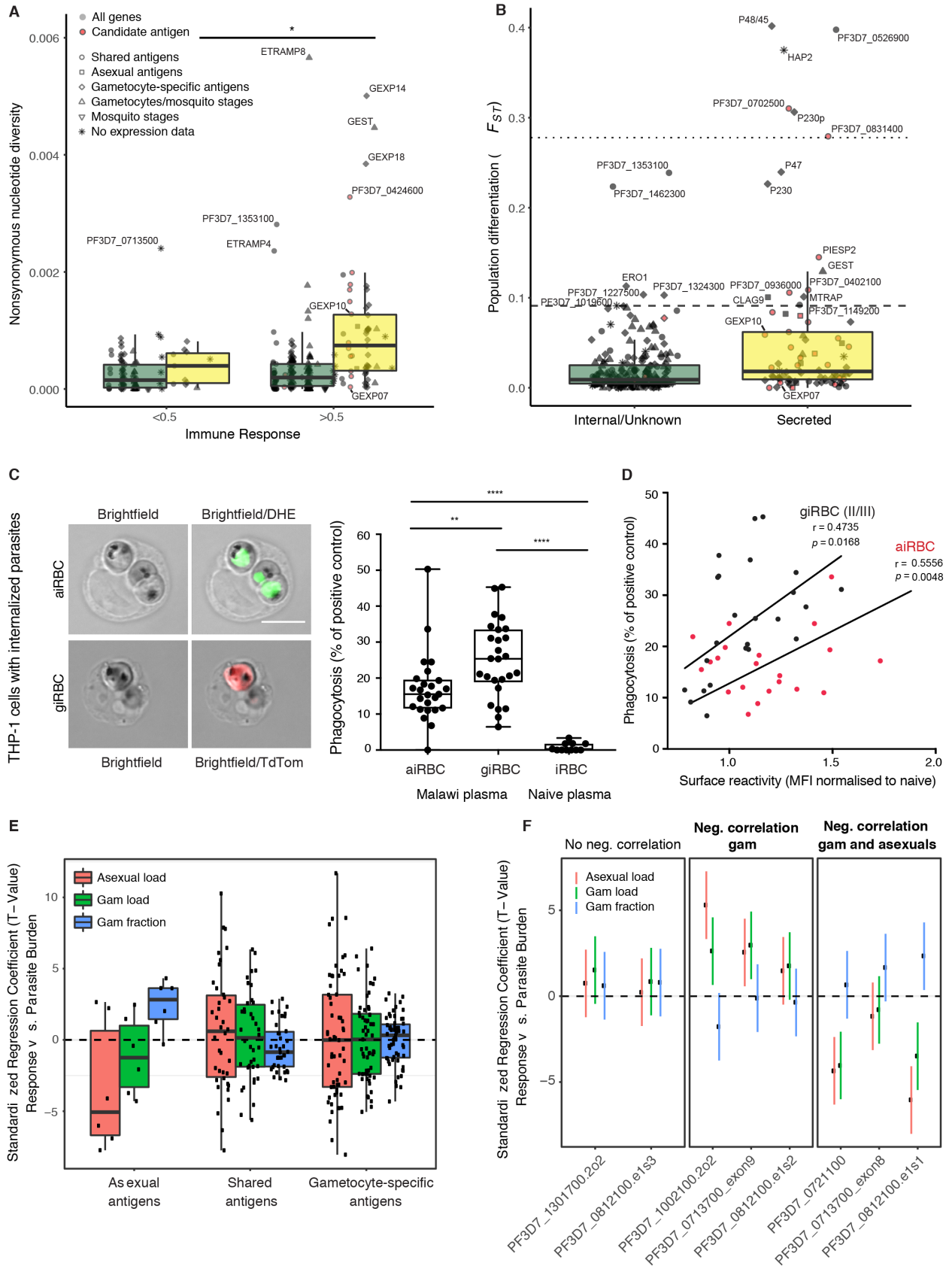


Figure 6: Candidate gametocyte surface antigens elicit responses correlated with reduced gametocyte burden and a subset show minimal genetic diversity.

A. Nonsynonymous nucleotide diversity for all antigens present on the protein array, stratified by stage, localization and level of immune response (Mann-Whitney U Test, $p < 0.05$). Genome data are from a set of parasite samples in Senegal. **B.** Population differentiation between Senegal and Malawi parasite samples for secreted and internal proteins (F_{ST} at nonsynonymous sites; Mann-Whitney U Test, $p = 2.0 \times 10^{-5}$). The dotted and dashed lines mark the 99th and 95th percentile of genome-wide nonsynonymous F_{ST} values. **C.** Left panel: internalized aiRBCs and giRBCs upon phagocytosis by THP-1 cells. aiRBCs are stained with the nuclear dye dihydroethidium (DHE) and giRBCs show TdTomato (TdTom) reporter fluorescence. Right panel: phagocytosis index of Malawi plasma samples relative to positive control (rabbit anti-human RBC) and naive US serum. **D.** iRBC phagocytosis vs. surface recognition. **E.** Associations were estimated between gametocyte fraction (gametocytes/total parasites), gametocyte and asexual parasite load, and secreted antigen fragment responses by peptide array (after normalization to IVTT controls and quantile normalization as in **Figure 1**) across 3 malaria-exposed populations. Median standardized regression coefficient -0.86, Wilcoxon test $p=0.087$. **F.** Regression coefficients (and 95% confidence intervals) between individual protein fragments of the prioritized candidate antigens and parasite parameters (either gametocyte fraction, gametocyte load or asexual parasite load). Fragments are stratified by their correlation with parasite parameters. Significance values: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

918 **Tables:**

Age (years)		Gender		Location		RDT status	
≤5	106 (43.44%)	Male	102 (41.8%)	Chikhwawa	171 (70.1%)	+	169 (69.3%)
>5,≤12	28 (11.48%)	Female	142 (58.2%)	Ndirande	35 (14.3%)	-	75 (30.7%)
>12,<30	47 (19.26%)			Thyolo	38 (15.6%)		
≥30	63 (25.82%)						
Total							244

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920 **Table 1. Characteristics of Malawian study population.** Chikhwawa has year-round malaria

921 transmission whereas Ndirande and Thyolo have more seasonal transmission peaking during the rainy

922 season each year. RDT=rapid diagnostic test.

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Accession ID	Export motif	Protein description	Stage annotation	Previously described localizations	Detection method	Host phenotypes	Conservation	Validated surface expression
PF3D7_0601900	PNEP	Conserved Plasmodium protein, unknown function	Shared	Maurer's clefts	Proteomics		Pf	
PF3D7_0713700	NO	Conserved Plasmodium protein, unknown function	Gam/Mo	Unknown	Proteomics	Neg correlation with asex./gam. load	Pf, Pv, Pb	
PF3D7_0721100	PEXEL	Conserved Plasmodium protein, unknown function	Shared	RBC surface	Proteomics Array/flow correlation	pos. correlation with age/neg. correlation with asex./gam. load	Pf, Pv, Pb	
PF3D7_0812100	NO	Conserved Plasmodium protein, unknown function	Shared	Unknown	Proteomics	pos. correlation with age/neg correlation with asex./gam. load	Pf, Pv, Pb	
PF3D7_0831400	PEXEL	Plasmodium exported protein, unknown function (Hyp12)	Shared	Unknown	Proteomics		Pf	
PF3D7_1002100	PEXEL	EMP1-trafficking protein (PTP5)	Shared	Maurer's clefts	Proteomics		Pf	X
PF3D7_1038000	NO	Antigen UB05	Shared	Unknown	Proteomics		Pf, Pv, Pb	
PF3D7_1301700	PEXEL	Plasmodium exported protein (hyp8), unknown function (GEXP07)	Shared	RBC surface	Proteomics/ Mouse sera array (+trypsin enriched)		Pf	X

927 **Table 2: Prioritized candidate gametocyte antigens.** 8 candidate gametocyte antigens were identified by:

928 1) predicted or known host secretion, 2) proteomics of trypsin-treated and –untreated giRBC membranes, 3)
 929 correlations between plasma reactivity by protein array and flow cytometry, 4) array reactivity of serum from
 930 mice immunized with trypsin-treated and –untreated giRBC membranes, 5) exposure-dependent increase of

931 IgG in malaria-positive individuals, 6), negative correlation with asexual and gametocyte load, and 7) low
932 genetic diversity and divergence. 3 candidates (marked in bold) fulfill all criteria (1-6). Previously described
933 stage annotation and localization was retrieved from plasmodb.org (21, 23, 24, 53). Further details on
934 candidates are provided in **table S7** and for 3 top candidates in **fig. S8**. Pf=*P. falciparum*; Pv=*P. vivax*,
935 Pb=*P. berghei*.

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